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Third-generation cephalosporin resistance conferred by a chromosomally encoded *bla*$_{CMY-23}$ gene in the *Escherichia coli* ST131 reference strain EC958

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Objectives: *Escherichia coli* ST131 is a globally disseminated MDR clone originally identified due to its association with the *bla*$_{CTX-M-15}$ gene encoding an ESBL. It is thus assumed that *bla*$_{CTX-M-15}$ is the major determinant for resistance to β-lactam antibiotics in this clone. The complete sequence of EC958, a reference strain for *E. coli* ST131, revealed that it contains a chromosomally located *bla*$_{CMY-23}$ gene with an upstream IS*Ecp1* element as well as several additional plasmid-encoded β-lactamase genes. Here, we examined the genetic context of the *bla*$_{CMY-23}$ element in EC958 and other *E. coli* ST131 strains and investigated the contribution of *bla*$_{CMY-23}$ to EC958 resistance to a range of β-lactam antibiotics.

Methods: The genetic context of *bla*$_{CMY-23}$ and its associated mobile elements was determined by PCR and sequencing. Antibiotic susceptibility testing was performed using Etests. The activity of the *bla*$_{CMY-23}$ promoter was assessed using *lacZ* reporter assays. Mutations were generated using λ-Red-recombination.

Results: The genetic structure of the IS*Ecp1*-IS*5*-*bla*$_{CMY-23}$ mobile element was determined and localized within the *betU* gene on the chromosome of EC958 and five other *E. coli* ST131 strains. The transcription of *bla*$_{CMY-23}$, driven by a previously defined promoter within IS*Ecp1*, was significantly higher than other β-lactamase genes and could be induced by cefotaxime. Deletion of the *bla*$_{CMY-23}$ gene resulted in enhanced susceptibility to cefoxitin, cefotaxime and ceftazidime.

Conclusions: This is the first known report to demonstrate the chromosomal location of *bla*$_{CMY-23}$ in *E. coli* ST131. In EC958, CMY-23 plays a major role in resistance to third-generation cephalosporins and cephamycins.

Keywords: *E. coli* ST131, antibiotic resistance, AmpC β-lactamases

Introduction

Escherichia coli ST131 is a globally disseminated MDR clone originally identified due to its close association with the spread of the *bla*$_{CTX-M-15}$ ESBL gene.[1–3] Two studies have recently reported the global epidemiology of *E. coli* ST131 using WGS, and revealed the *bla*$_{CTX-M-15}$ allele is highly prevalent within a fluoroquinolone-resistant FimH30 (*H*30) ST131 sublineage (referred to as clade C2[4] or *H30*-Rx[5]). We have characterized and completely sequenced the reference ST131 strain EC958.[6] *E. coli* EC958 is a urinary tract infection (UTI)-derived fluoroquinolone-resistant, *fimH*30 *E. coli* ST131 strain.[7] The strain is a representative member of the UK epidemic strain A, one of the major pathogenic lineages causing UTI across the UK,[3] and belongs to the recently defined phylogenetic C2 lineage of ST131.[4] EC958 contains a large multidrug resistance plasmid (pEC958; HG941719) that harbours a copy of *bla*$_{CTX-M-15}$ gene. The *bla*$_{CTX-M-15}$ gene has always been seen with an upstream IS*Ecp1* insertion element.[8] IS*Ecp1* contains a well-described outward-facing promoter that drives the transcription of the downstream *bla*$_{CTX-M-15}$ gene,[9] and this transposition unit has been identified on multiple plasmids characterized from ST131 strains.[10] Several different insertions of IS*26* in the region upstream of *bla*$_{CTX-M-15}$ have been reported, including the insertion at 24 bp upstream of the right inverted repeat (IRR) of IS*Ecp1*, which results in reduced resistance to cephalosporins.[10] This particular insertion was first identified in UK epidemic strain A isolates and was also found on plasmid pEC958 from the *E. coli* ST131 strain EC958.

In addition to the production of ESBLs, resistance to third-generation cephalosporins can also be mediated by chromosomal and plasmid-encoded AmpC (or class C) β-lactamases.[11] The plasmid-encoded *bla*$_{CMY-1}$ gene and its variants are related to chromosomal AmpC enzymes from *Aeromonas* spp., while

bla_{CMY-2} and its variants are related to AmpC β-lactamases from *Citrobacter freundii*.[11] bla_{CMY-2} gene is the most frequently reported plasmid-mediated AmpC β-lactamase worldwide; it confers resistance to cephamycins (cefoxitin and cefotetan) and oxyimino-cephalosporins (cefotaxime and ceftazidime) and is poorly inhibited by β-lactamase inhibitors, including clavulanate, sulbactam and tazobactam.[12] Several studies have reported the identification of bla_{CMY-2} in *E. coli* ST131.[13,14] In addition, a variant of bla_{CMY-2}, named bla_{CMY-23}, has been identified in several *E. coli* ST131 isolates belonging to the UK epidemic *E. coli* strain A.[15] The bla_{CMY-2} and bla_{CMY-23} genes differ by a single nucleotide, which results in a Glu239Gly amino acid substitution in CMY-23.

A bla_{CMY-23} gene accompanied by an upstream IS*Ecp1* insertion element was found inserted in the chromosome of EC958. This led us to hypothesize that CMY-23, instead of CTX-M-15, is the β-lactamase responsible for cephalosporin resistance in EC958. Here, we investigated the contribution of bla_{CMY-23} to EC958 resistance to a range of β-lactam antibiotics, and demonstrated that it is the primary mediator of resistance to third-generation cephalosporins and cephamycins.

Materials and methods

Bacterial strains and growth conditions

The strains used in this study are listed in Table S1 (available as Supplementary data at *JAC* Online). All *E. coli* strains were routinely cultured at 37°C on solid or in liquid Lysogeny Broth (LB) medium supplemented with an appropriate antibiotic: chloramphenicol (30 mg/L), gentamicin (20 mg/L) or ampicillin (100 mg/L).

Antimicrobial susceptibility testing

The MICs of β-lactam antibiotics were determined by Etest (bioMérieux Australia) on Mueller–Hinton agar at 37°C using EUCAST breakpoints (http://www.eucast.org/clinical breakpoints/).

Molecular methods

Genomic DNA preparation and DNA sequencing were performed as described previously.[16] Oligonucleotides used in this study are listed in Table S2. Plasmids pQF50::IS*Ecp1* and pQF50::IS*5*+IS*Ecp1* were created by PCR amplification of the fragments indicated in Table S1 and cloned into pQF50 via BamHI–HindIII digestion. The resulting plasmids were electroporated into *E. coli* TOP10. Mutants containing deletions in the bla_{CMY-23} gene and IS*5* were constructed by λ-Red-mediated recombination as previously described.[7] β-Galactosidase assays were performed as previously described.[16]

Results and discussion

The bla_{CMY-23} gene is located on the chromosome of *E. coli* EC958

E. coli EC958 contains a chromosomally encoded bla_{CMY-23} gene (EC958_4781). Closer inspection of its genomic context suggests an insertion event of a 4277 bp mobile element (nucleotides 4937433–4941709, including IS*Ecp1*, IS*5* and bla_{CMY-23}) into the *betU* gene (encodes a betaine uptake system) within the GI-*leuX* genomic island, creating 5 bp direct repeats (DRs) of TATAT (Figure 1a). This insertion was not found in the *E. coli* ST131 strain JJ1886 (CP006784), which harbours two tandem copies of the GI-*leuX* island (Figure S1). IS*Ecp1* is known to mediate the transposition of its downstream genes using an imperfect IRR (IRR′).[17] Indeed, we identified an IRR′ with the sequence 5′-gtGTAGctcCccGa-3′ (nucleotides matching the IS*Ecp1* IRR are capitalized) immediately before the right DR. The IS*5* element is located 152 bp before the end of IS*Ecp1* (Figure 1b and c) and flanked by a 4 bp DR of TTAA. This location does not interrupt the IS*Ecp1* transposase gene and leaves the promoter region at the end of IS*Ecp1* intact. It is not clear whether this IS*5* insertion occurred prior to or after the movement of IS*Ecp1*-bla_{CMY-23} to the chromosome.

Prevalence of ISEcp1-bla_{CMY-23}

The bla_{CMY-23} gene (DQ438952) was first reported from isolates within the UK epidemic strain A, which was later shown to be part of the *E. coli* ST131 lineage.[15] A second bla_{CMY-23} sequence has been reported from *Salmonella enterica* serovar Senftenberg (DQ463751). However, neither of these entries includes information about the genomic context nor location of the bla_{CMY-23} gene.

Analysis of a global collection of previously sequenced ST131 strains[4] identified five clade C2 strains (S30EC, accession number ERR161246; S39EC, accession number ERR161248; S43EC, accession number ERR161249; S47EC, accession number ERR161250; and S53EC, accession number ERR161251) that possess the bla_{CMY-23} gene. Further PCR and sequencing of S30EC, S39EC and S43EC confirmed that they contain an identical IS*Ecp1*-IS*5*-bla_{CMY-23} sequence inserted at the same location on the chromosome as in EC958 (Figure 1b). Of note, EC958 and these five strains were all isolated from the same hospital in the UK (from 2004 to 2007) where the first bla_{CMY-23} positive strain was reported.[15]

We also identified one strain (S79EC, accession number ERR161305) from the ST131 clade A phylogeny that contained the bla_{CMY-2} gene within a transposition unit identical to that found in IncI1 plasmids pSTM709 (HG428759) and pCVM29188_101 (CP001121). This unit lacks IS*5*, has a longer region downstream of IS*Ecp1* (2253 bp compared with 1422 bp) and based on its flanking genes may be located on a plasmid in S79EC (Figure 1b).

The bla_{CMY-23} gene is the major contributor to EC958 resistance to third-generation cephalosporins

EC958 harbours several genes that could contribute to its resistance to β-lactam antibiotics, including bla_{TEM-1}, bla_{OXA-1}, $bla_{CTX-M-15}$, bla_{CMY-23} and the chromosomal *ampC* gene. To specifically demonstrate the contribution of bla_{CMY-23} to EC958 resistance, we mutated the bla_{CMY-23} gene using λ-Red-mediated homologous recombination. The resultant strain, designated EC958Δbla_{CMY-23}, was then tested in parallel with WT EC958 against a panel of β-lactam antibiotics. Despite the presence of pEC958 (which harbours bla_{TEM-1}, bla_{OXA-1} and $bla_{CTX-M-15}$), EC958Δbla_{CMY-23} displayed significantly enhanced susceptibility to cefoxitin, cefotaxime and ceftazidime (8–16-fold reduction in MIC; Table 1) compared with WT EC958. Taken together, our data confirm that the bla_{CMY-23} gene is the main determinant that mediates resistance to second- and third-generation cephalosporins in EC958.

bla_{CMY-23} expression is induced by cefotaxime, but not affected by IS5

We used qRT–PCR to evaluate the transcription of the five β-lactamase genes in EC958. Our results showed that bla_{CMY-23}

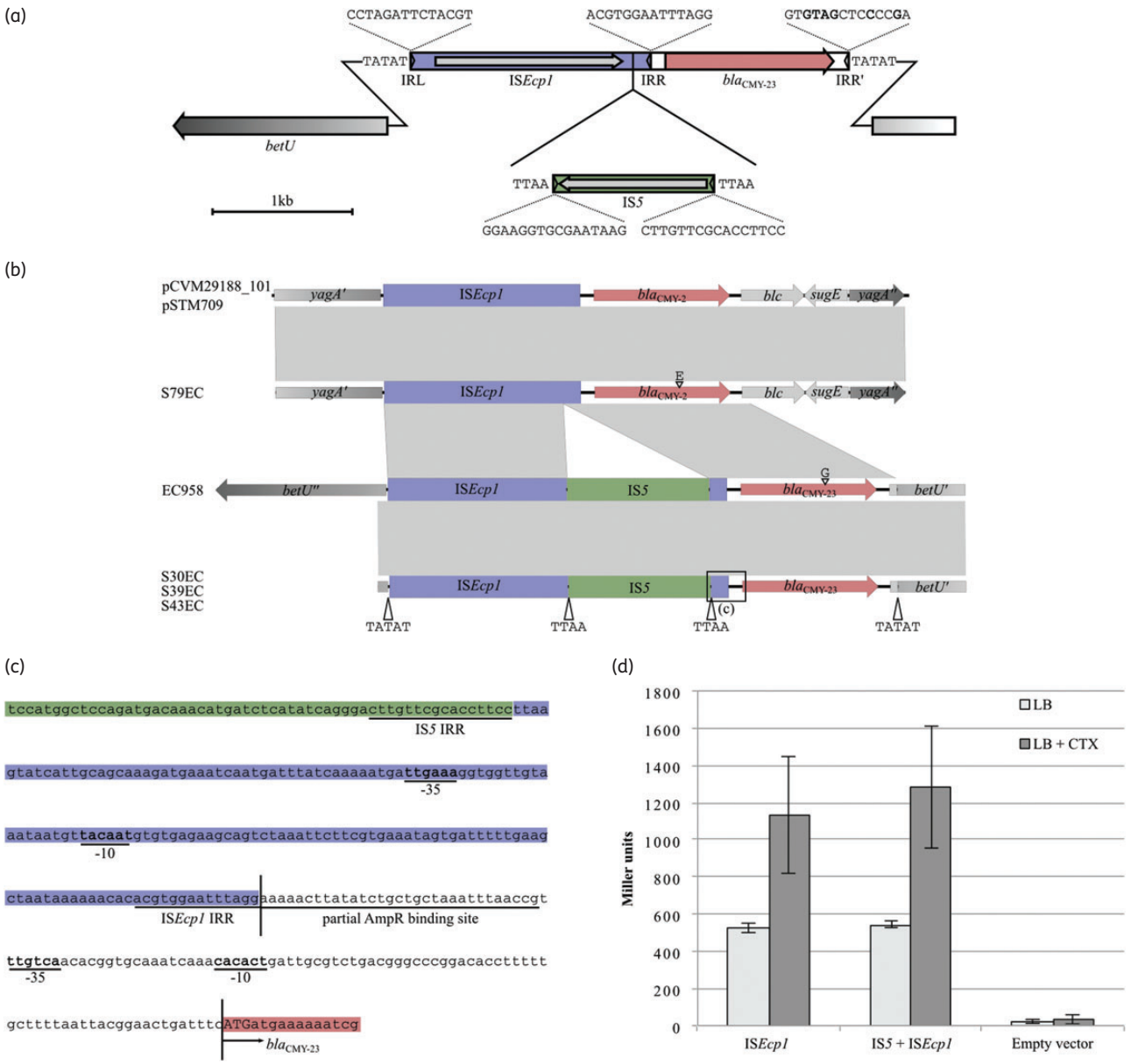

Figure 1. The chromosomal location of bla_{CMY-23} in EC958 and other *E. coli* ST131 strains. (a) The insertion of IS*Ecp1* (purple), IS*5* (green) and bla_{CMY-23} (red) into the *betU* gene. The sequences of DRs and inverted repeats supporting putative transposition events are shown. (b) Sequence comparisons showing the genomic context of bla_{CMY-23} on the chromosome of ST131 strains and the closely related bla_{CMY-2} on IncI1 plasmids. The grey shadow connecting different strains indicates 100% similarity except for one single nucleotide change in bla_{CMY-2} resulting in an E to G amino acid change in CMY-23 (indicated by arrow heads with the respective amino acid code). The boxed sequence is shown in full in panel (c). (c) The sequence of the bla_{CMY-23} promoter region showing the −10 and −35 promoter consensus sites provided by IS*Ecp1* (underlined within the purple region), as well as the *C. freundii*-derived partial AmpR binding site and associated promoter sequence. (d) β-Galactosidase assays showing the activity of the IS*Ecp1* promoter and its induction in the presence of cefotaxime. β-Galactosidase activity is expressed as Miller units. CTX, cefotaxime; IRL, left inverted repeat. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

is the most strongly transcribed β-lactamase gene in EC958 (8-fold higher than the reference gene *recA*; Figure S2). In contrast $bla_{CTX-M-15}$ is transcribed weakest (12-fold lower than *recA*). These data are consistent with the mutant data reported above, and in accordance with previous publications that show IS*Ecp1* contains a functional promoter that drives the transcription of downstream genes including CMY variants.[9,18]

To investigate the activity of the bla_{CMY-23} promoter in EC958, we cloned the region corresponding to the end of IS*Ecp1* in front of the promoterless *lacZ* gene in plasmid pQF50 to generate plasmid pQF50::IS*Ecp1*. A 2-fold increase in β-galactosidase activity was observed for *E. coli* TOP10 harbouring pQF50::IS*Ecp1* grown in LB medium containing cefotaxime compared with LB medium alone ($P < 0.0001$) (Figure 1d). No β-galactosidase

Table 1. Antimicrobial susceptibility (expressed as MIC in mg/L) of EC958 WT and mutant strains

Antibiotic	EC958	EC958Δbla_{CMY-23}	EC958ΔIS*5*
Ampicillin	≥256 (R)	≥256 (R)	≥256 (R)
Amoxicillin/clavulanic acid	24 (R)	12 (R)	16 (R)
Piperacillin	≥256 (R)	≥256 (R)	≥256 (R)
Piperacillin/tazobactam	16 (I)	12 (I)	16 (I)
Cefoxitin	48 (NA)	4 (NA)	48 (NA)
Cefotaxime	12 (R)	1.5 (I)	12 (R)
Ceftazidime	16 (R)	1 (S)	16 (R)
Cefpirome	3 (NA)	1 (NA)	3 (NA)
Aztreonam	2 (I)	1 (S)	2 (I)

R, resistant; I, intermediate; S, susceptible; NA, breakpoints not available.

activity was detected in the vector control strain grown under the same conditions.

IS*5* has been shown to alter transcription by enhancing the activity of a promoter downstream of its site of insertion.[19,20] Thus, we investigated whether the IS*5* element located within IS*Ecp1* affects expression of the bla_{CMY-23} gene using two complementary approaches. First, we cloned the IS*Ecp1* promoter region together with the upstream IS*5* in front of the promoterless *lacZ* gene in plasmid pQF50 to generate plasmid pQF50::IS*5*+IS*Ecp1* and showed that there was no difference in the β-galactosidase activity between TOP10(pQF50::IS*5*+IS*Ecp1*) and TOP10(pQF50::IS*Ecp1*) (Figure 1d). Second, we deleted the IS*5* element using λ-Red-mediated homologous recombination. The resultant strain, designated EC958ΔIS*5*, did not show any difference in antibiotic resistance profile from that of WT EC958 (Table 1). These results demonstrate that the bla_{CMY-23} promoter provided by IS*Ecp1* is functional and can be induced by cefotaxime.

Conclusions

$bla_{CTX-M-15}$ is considered to be the main determinant for resistance to third-generation cephalosporins in *E. coli* ST131. Here, we showed that in EC958, which contains a plasmid-located $bla_{CTX-M-15}$ gene, cephalosporin resistance is conferred by a chromosomal bla_{CMY-23} gene that encodes an AmpC type β-lactamase. We also showed that the bla_{CMY-23} in EC958 is strongly transcribed from a promoter located within IS*Ecp1* that can be induced by cefotaxime.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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